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13. ABSTRACT (Maximum 200 Words)

Goals met during the first funding period of this grant include developing methodologies to measure cellular polyphosphophosphate concentrations in breast cancer cells and the preparation of breast cancer cell lines with altered levels of polyphosphate. Specifically, a reproducible and quantitative procedure to extract and enzymatically measure polyphosphate concentrations in breast cancer cells is now in hand. Additionally, a plasmid containing a copy of the *S. cerevisiae* exopolyphosphatase (scPPX) gene behind a constitutively active CMV promoter was constructed and successfully stably transfected into breast cancer cell lines. scPPX degrades polyphosphate by processively removing the terminal phosphate moiety from the polyphosphate polymer. Thus, the presence of extra copies of scPPX results in depleted levels of cellular polyphosphate. These accomplishments of the first funding period provide the necessary tools to investigate the link between cellular polyphosphates and breast cancer, as outlined in the proposal.

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INTRODUCTION

Cellular polyphosphates (polyP), dynamic long linear polymers of orthophosphate linked by high-energy phosphoanhydride bonds, are found at micromolar levels in every organism and tissue examined. The polymers are very dynamic, being continuously synthesized and degraded. Their length can be as short as three phosphates to as long as one thousand phosphate moieties. There have been a number of functions suggested for polyP. Some have been examined experimentally while others are based on the compound's structure and anionic property. These functions include polyP serving as: an ATP substitute and energy source, a reservoir of orthophosphates, a chelator of divalent metals, a buffer against alkali, a channel for DNA entry into cells, and a regulator of stress response and cell survival (1).

An understanding of the role of polyphosphates in cellular physiology began to emerge with the identification of the genes and corresponding enzymes involved in polyP synthesis and degradation. The biosynthetic enzyme, polyphosphate kinase (PPK) has been purified from *Escherichia coli* (*E. coli)* (2), as have an exopolyphosphatase (PPX) and endopolyphosphatase (PPN), which also have been isolated from *S. cerevisiae*. Cells with genetically altered levels of polyP have been created by deleting or overexpressing the genes encoding PPK and PPX (3, 4, 5). The purified enzymes have also proven to be invaluable tools for developing sensitive and reliable assays to measure polyP levels in different organisms and tissues (6).

In prokaryotes, accumulating data indicate that polyP promotes cell survival. Reduced polyP levels compromise how the cells respond to and survive environmental challenges, such as nutrient deprivation, heat shock, phosphate deficiency, oxidative stress, and osmotic challenge. Relatedly, polyP levels have been observed to transiently increase following exposure to such conditions. While these general characteristics regarding polyP have been determined for micro-organisms, few studies have examined polyphosphate concentration and location in mammalian cells. Kumble and Kornberg found levels of 25 to 120 μM (in terms of P_i residues) in rodent tissues (brain, heart, kidneys, liver, and lungs) and specific subcellular fractions (nuclei, mitochondria, plasma membranes, and microsomes). The synthesis of polyphosphate from P_i by fibroblasts, T-cells, kidney, and adrenal cell lines attained levels of excess of 10 pmol per mg of cell protein per hour. Further tests showed the turnover of polyphosphate varied from one hour in adrenal cells to over four hours in fibroblast cells. They concluded that the ubiquity of polyphosphate and variations in its polymer length, location, and metabolism are indicative of a multiplicity of functions for this polymer in mammalian systems. (7)

A direct relation between breast cancer and polyphosphate has been observed in one study. MCF-7 cells depleted of polyphosphate by overproduction of scPPX did not grow in serum-free media, while MCF-7 cells without overproduction of PPX grew normally to confluency. Polyphosphate was also found to stimulate M-Tor, but not other related DNA protein kinases. Interestingly, M-Tor is part of a signaling pathway that activates anti-apoptosis proteins (8). The research, described in this summary report and supported by the Department of Defense, has allowed the relationship between breast cancer and polyphosphates to be further studied while contributing to the education and training of a doctoral student.

During the first year of this research program, the tasks included developing procedures to

extract and quantitatively measure polyP concentrations in breast cancer cells and to create breast cancer cell lines with depleted levels of polyP.

In order to gather the polyP from the breast cancer cells, the cell pellet must first be thoroughly lysed and treated with RNase, DNase, Proteinase K and SDS to degrade the nucleic acid and disrupt protein interactions. The lysate is combined with a slurry of a silica compound which binds with anionic chains. Since the nucleic acid is already degraded, polyP is the only anionic polymer remaining to bind to the silica. Once the polyP is washed and eluted from the silica, it is transferred into ATP through the PPK reverse reaction. One ATP is produced from each phosphate in the polyP chain. ATP can then be measured by the quantity of light produced in a luciferase reaction.

A plasmid was constructed containing a copy of the *S. cerevisiae* exopolyphosphatase (scPPX) gene behind a constitutively active CMV promoter. scPPX rapidly degrades polyP by processively liberating the end phosphate moiety from the polymer. Therefore, the addition of extra copies of scPPX into a cell will decrease its polyphosphate levels. The constructed plasmid was stably transfected into breast cancer cell lines MCF-7. Differences in polyphosphate levels between these new cells and the original, unmodified breast cancer cell will be confirmed.

With these accomplishments, the research outlined in the proposal can advance to the goals of examining the how the lack of polyP affects breast cancer growth and survival, cell cycle, and gene expression.

KEY ACCOMPLISHMENTS

Research Accomplishments

- Designed and optimized a procedure to extract polyphosphates from breast cancer cells with excellent and reproducible yield.
- Constructed a mammalian expression plasmid containing *S. cerevisiae* exopolyphosphatase (scPPX) gene behind a constitutively active CMV promoter
- Performed DNA transfection of the constructed plasmid into MCF-7 breast cancer cells
- Selected thirty-six clones of MCF-7 cells stably transfected with scPPX
- Screened half of the stable transfectants to determine the existence of scPPX in the chromosome

Training Accomplishments

- Participated in numerous seminars concerning breast cancer associated with the Lombardi Comprehensive Cancer Center
- Participated in the Breast Cancer Symposium hosted by Georgetown University on December 6, 2004
- Enhanced skills in tissue culture, DNA transfection, plasmid design and development, and genetic analysis

REPORTABLE OUTCOMES

- Georgetown University Department of Biochemistry and Molecular Biology Graduate Student Data Presentation – "Role of Inorganic Polyphosphate in Enhancing Survival after UV Induced DNA Damage in *Escherichia coli"* November 1, 2004
- Georgetown University Biomedical Research Days Poster Exhibition – "Role of Cellular Polyphosphate in Enhancing Survival after UV Induced DNA Damage in *Escherichia coli"* February 24, 2004
- Application for a Susan G. Komen Breast Cancer Foundation – Basic Clinical and Transduction Grant - "Cellular Polyphosphates and Breast Cancer Response to Chemotherapeutic and Radiation Treatments" P.I. Elliott Crooke Ph.D.
- MCF-7/PPX – A new breast cancer cell line originating from MCF-7 cells with a genomic copy of *ppx* controlled by a CMV promoter
- A protocol for the extraction and measurement of polyphosphate from breast cancer cells

PROJECT CHALLENGES

- A protocol for extracting and measuring polyphosphate levels in bacteria had been developed prior to this research. It was assumed that this protocol could be easily adjusted to accommodate mammalian cells. Unfortunately, separation of polyphosphate from the cell lysate was more difficult than expected and required numerous modifications to efficiently extract polyphosphates from the breast cancer cells.
- An antibody does not yet exist for scPPX and therefore it is not easily determined if scPPX is expressed in the transfected cells. Therefore in the future, the PPX in the plasmid may be fused to a tag to allow detection using commercially available antibodies to the tag.
- Stably transfected MCF-7 cells often lose the enzyme activity of the inserted gene after multiple passages. This appears to limit the time that the tissue cultures can be used for survival assays.

PROJECT OPPORTUNITIES

- The mechanisms of how polyphosphate interact with other cellular components continue to be studied in *E. coli* and other bacteria. The simplicity of these organisms makes it easier to detect phenotypic and genotypic differences in cells caused by polyphosphate, allowing the identification of cellular interactions. Findings from these model systems will be used to predict and guide the work performed in the more complex breast cancer cells, specifically with regard to how polyphosphates may affect the cell cycle and gene expression.

CONCLUSIONS

The recipient of this pre-doctoral training grant from the Department of Defense continues to not only build on the knowledge of polyphosphate and breast cancer, but also in her training as a biochemical scientist preparing for a career in breast cancer research. Due to the novel idea of exploring polyphosphate and breast cancer, none of the tools or materials existed prior to the start of this research. Over the past year, the investigator has acquired the methodologies, reagents, and cell lines necessary to begin analyzing the relationship between polyphosphates and breast cancer cell biology.

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